

APPLICATION FOR LETTERS PATENT

**Titl : R ceptor-Mediat d Uptake of P ptid s That Bind the Human
Transferrin R c ptor**

5 This work was supported by USPHS grants R01 HL58339 and IP50
DE/CA 11910 and by NCI grant CA-13148. Hence, the United States
Government has certain rights in this invention.

This application takes priority from U.S. Provisional patent
application 60/253,940.

10 Field of the Invention:

This invention relates to use of peptides which target the
human transferrin receptor. Peptides of the invention can be used
to direct other peptides, proteins and other diagnostic or
therapeutic agents into cells for both diagnostic and therapeutic
15 purposes.

Background of the Invention:

Previous work relating to redirecting viral vectors in gene
therapy by using short peptide ligands to redirect virus particles
to specific cell types are known. One of the limitations of this
20 strategy is that short peptide sequences that bind efficiently to
cell surface receptors on specific cell types must be identified.
One experimental approach to identify such short peptides that
holds promise is bacteriophage display.

For more than a decade, phage display has exploited the
25 physical linkage between random peptide sequences expressing on
phage and the DNA encoding that sequence. This linkage allows for
rapid identification of peptide ligands. A random peptide sequence

is expressed as a fusion with a bacteriophage coat protein and is available for testing as a ligand for various targets. Phage display has successfully been used to identify single chain antibodies with specificity for various biological molecules.

5 Phage display strategies can be used to elucidate the amino acids responsible for protein-protein interactions, to find organ-specific phage, and to find substrate recognition sequences for enzymes. The process of using multiple rounds of phage display to enrich for a particular sequence is called biopanning.

10 The human transferrin receptor (hTfR) has been studied extensively as a model system for receptor-mediated endocytosis, a marker for cellular proliferation, and a target for therapeutics. The hTfR is ubiquitously expressed and over-expressed at least 100 fold in oral, liver, pancreatic, prostate and other cancers. This

15 increase in transferrin receptor (TfR) in cancers has been attributed to the increased metabolism of these transformed cells, making the hTfR a useful diagnostic marker. Because of its expression pattern and pathway characteristics, the hTfR is an attractive target for therapeutics. The TfR is a dimer composed of

20 two identical 95 kDa subunits and is responsible for the majority of cellular iron uptake. The type II cell surface receptor binds 80 kDa transferrin (Tf) and the complex is internalized through clathrin-coated pits. Iron is released from transferrin in the acidic early endosome and the apotransferrin-receptor complex is

25 recycled back to the cell surface where apotransferrin is recycled.

A blast search failed to yield any significant homologies

between either HAIYPRH (Seq. ID No. 1) or THRPPMWSPVWP (Seq. ID No. 2) to known proteins, including Tf.

Summary of the Invention:

This invention relates to peptides which are capable of binding to and internalizing with the human transferrin receptor (hTfR). The sequences HAIYPRH (Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2) are capable of binding to and internalizing with the human transferrin receptor. When these molecules were fused with other molecules, the fusion product was internalized in cells expressing hTfR. The sequences have use for targeting other peptides and proteins into cells expressing hTfR. The phage display system using whole cell selective biopanning could also be applied to find small ligands for other cell surface receptors. This sequence is not found in human transferrin protein. Furthermore, this sequence does not compete with transferrin itself for binding to the hTfR.

Detailed Description of the Invention:

It is important that easily produced peptides that can facilitate entry of diagnostically and therapeutically useful peptides and proteins into cells having particular characteristics be available. The identification of peptides that will facilitate entry of such peptides into cells which are more likely to be aberrant has particular use. The peptides of the invention are useful for facilitating entry of diagnostically and therapeutically useful agents, including peptides and proteins. Since malignant cells produce increased expression of hTfR, the peptides, HAIYPRH

(Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2), are particularly useful for study and treatment of malignancies.

A phage display selection strategy was utilized that resulted in identification of the peptides. This selection system is based on alternating rounds of negative selection on chicken embryo fibroblast (CEF) cells lacking hTfR and positive selection on chicken embryo fibroblast cells expressing hTfR (CEF+hTfR). Biopanning on whole cells was exploited to select the peptides HAIYPRH (Seq. ID No. 1) and THRPPMWSPVWP (Seq. ID No. 2). These peptides were able to target a macromolecule to and internalize through the hTfR, as was demonstrated by phage binding, competition and immunofluorescence studies. It was also shown that these two peptides bind sites that do not overlap with the native ligand, transferrin, indicating they could be used in vivo for targeting macromolecules to the endocytic pathway in hTfR-positive cells.

The biopanning procedure could be applied to find small peptide ligands for other cell surface receptors. There is a great need to find new epitopes on various cancer cell types for diagnostic purposes. The subtractive method of biopanning disclosed herein would be useful for finding new cell surface markers. Biopanning on whole cells can be especially useful in situations where the receptor can not be purified or does not maintain its native confirmation when isolated.

Materials and Methods:

Cell lines: The two chicken embryo fibroblast cell lines, CEF and CEF+hTfR, used for selective biopanning, were described

previously (Collawn, et al, Cell, 63, 1061-1072 (1990) and Odorizzi, et al., J. Cell Biol., 126, 317-330 (1994)). Chicken embryo fibroblasts have been used extensively for study of hTfR. The native cells express chicken transferrin receptors, but this receptor cannot bind human transferrin. Two cell lines were previously established through stable transduction with retroviral vectors to yield CEF and CEF+hTfR cells. CEF cells do not express the human transferrin receptor. CEF+hTfR cells constitutively express hTfR. Protein expression of hTfR by CEF cells was periodically checked by ¹²⁵I-Tf binding. Both cells are grown in monolayer cultures in Dulbecco's Modified Eagle Medium supplemented with 1% chicken serum, 1% bovine calf serum, 1% L-glutamine 200 nM, and 2% tryptose phosphate and maintained at 37°C in 13% CO₂.

Antibodies: Monoclonal anti-GFP (green fluorescent protein) antibody (Clontech, Palo Alto, CA) was used for Western blot analysis and immunofluorescence at 1:5,000 and 1:250 dilution, respectively. Horse radish peroxidase conjugated goat anti-mouse antibody (Pierce, Rockford, IL), Oregon-Green and Texas-Red secondary antibodies (Molecular Probes, Eugene, OR) were used at 1:10,000, 1:250, 1:250 dilution, respectively.

Electrophoretic methods: Samples were dissolved on SDS-PAGE gels by the methods of Laemmli and transferred to nitrocellulose membrane by electroblotting for Western blot analysis (Laemmli, U.K, Nature, 227, 680-685). The membranes were blocked with 5% milk in tris buffered saline with 1% Triton X-100 (TBS-TX) (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1% Triton X-100), and incubated with

primary antibody in TBS-TX with 2.5% milk overnight at 4°C. The membranes were then washed in TBS-TX and incubated with peroxidase-conjugated secondary antibody and developed with the enhanced chemiluminescence (ECL) kit in accord with the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, England).

Biopanning: Ph.D.-7™ or Ph.D.-12™ Phage Display Peptide Library Kit (New England Biolabs, Inc, Beverly, MA) was used for biopanning on CEF and CEF+hTfR cells. The Ph.D.™ phage display peptide library is based on a combinatorial library of random 7 or 12 amino acid peptides fused to a minor coat protein of the filamentous coliphage M13. In separate studies, two different phage display peptide libraries were used to select for 7-mer and 12 mer peptide sequences that could bind the hTfR expressed on the surface of CEF+hTfR cells. Cells were washed and incubated in serum-free Opti-MEM (Gibco BRL Life Technologies, Gaithersburg, MD) at 37°C for 1 hour prior to all biopanning procedures. Phage binding was carried out at 4°C in serum-free Opti-MEM with 1×10^6 cell/3.5 cm well. Initial biopanning procedures applied 2×10^{11} phage to CEF cells for two hours; unbound phage were transferred to CEF+hTfR cells for 1 hour. Cells were washed 10 times with Opti-MEM, and bound phage was quickly eluted with low pH buffer (0.2M glycine-HCl, pH 2.2) and neutralized with 1M Tris-HCl, pH 9.1. Eluted phage were amplified in 20 ml Luria-Bertani medium (LB) containing E. coli ER2537 (for 7-mer phage) and ER2783 (for 12-mer phage) at 37°C. Phage from liquid cultures were obtained by clearing the

supernatant twice by centrifugation at 10,000 rpm for 15 minutes at 4°C, and precipitated with 1/6 volume of PEG/NaCl (10% polyethylene glycol-8000, 2.5M NaCl) at 4°C overnight. Phage pellets were suspended in 1 ml TBS (50 mM Tris-HCl, 150 mM NaCl), and
5 precipitated with PEG/NaCl for 1 hour. Amplified phage were resuspended with 200 µl TBS, 0.02% NaN₃, and these amplified phage were used for additional rounds of biopanning. After each round of biopanning, the final elutes were titrated, amplified in E. coli, and plated onto LB plates. The plates were incubated at 37°C
10 overnight. Individual plaques were subjected to plaque amplification, DNA purification, and DNA sequencing using a modified Sanger sequencing reaction (Sanger, et al., Proc Natl Acad Sci USA, 74, 5463-5467 (1977)) with the appropriate sequencing primers.

15 7-mer sequencing primer: 5'-TGGGATTTTGCTAAAAAC-3' (Seq. ID No. 5)

12-mer sequencing primer: 5'-GTATGGGATTTTGCTAAACAAC-3'

(Seq. ID No. 6)

Peptide Synthesis: The peptides HAIYPRH (Seq. ID No. 1), IRHPHYA (Seq. ID No. 3), THRPPMWSPVWP (Seq. ID No. 2), and PWRPSHPVWMPT
20 (Seq. ID No. 4) were synthesized on an Applied Biosystems Model 440 by means of the solid phase peptide synthesis procedure at the Peptide Synthesis Core Facility of the University of Alabama at Birmingham (UAB) Comprehensive Cancer Center. These peptides were purified by high pressure liquid chromatography, and the molecular
25 weights were confirmed by mass spectrometry.

Binding and Competition Studies: Purified phage populations were

amplified and were verified to be homogenous through DNA sequencing. Cells were prepared for binding as was described for biopanning procedures. Preparations of plaque-purified and titered phage (1×10^{11}) were incubated in serum-free Opti-MEM on either CEF or CEF+hTfR cells at 4°C for 1 hour. The cells were washed repeatedly with Opti-MEM and bound phage were eluted with low pH buffer and subsequently titered. In competition studies, holotransferrin (Calbiochem, La Jolla, CA) or synthesized peptides were added to CEF+hTfR cells prior to addition of the phage for 1 hour at 4°C. Multiple trials were completed and average titers and standard deviations determined. The titers determined on CEF+hTfR cells were divided by the titers determined on CEF cells and multiplied by 100 to yield fold over control data points.

Modified GFP Constructs: Transferrin from human serum, bovine serum albumin (BSA), and purified wild-type GFP (wtGFP) were obtained from Sigma (St. Louis, MO) and Clontech (Palo Alto, CA), respectively. The tagged GFP genes were generated by the PCR with template DNA Clontech's GFP vector. The PCR reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler for 30 cycles of 95°C, 1 minute; 55°C, 1 minute; and 72°C, 1 minute.

HAIYPRH-tagged GFP PCR oligonucleotides were:

Upstream: 5'-TCTAGATCTGATGAGTAAAGGAGAAGAA-3' (Seq. No. 7)

Downstream: 5'-TTAAAGCTTTTAATGGCGCGATAGATCGCATGTTTGT
AGAGCTCATCCATGCC-3' (Seq. No. 8)

THRPPMWSPVWP-tagged GFP PCR oligonucleotides were:

Upstream: 5'-TCTAGATCTGATGAGTAAAGGAGAAGAA-3' (Seq. No. 7)

Downstream: 5'-TAAAGCTTTTACGGCCACACCGGGCTCCACATCGGCGGG

CGGTGGGTTTTGTAGAGCTCATCCATGCC-3' (Seq. No. 9)

The PCR products were purified with the Qiagen Gel Extraction Kit (Qiagen, Valencia, CA), and cut with BglII and HindIII restriction enzymes (Roche, Nutley, NJ), and subcloned into the pET-32a(+) bacterial expression vector (Novagen, Madison, WI). The resulting expression vector was verified using a modified Sanger sequencing method. The tagged GFP expression plasmids were transformed into BL21/DE3 E. coli and expression was induced for 3-4 hours with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the culture O.D.₆₀₀ = 0.5. Cells were pelleted, then resuspended in phosphate buffer with 20 mM imidazole followed by passage through a French press at 10,000-15,000 psi. Cell lysates were passed over a PiTrap nickel column (Amersham Pharmacia Biotech, Piscataway, NY). The column was washed and finally eluted using an imidazole gradient. The purified protein was assayed by SDS-PAGE followed by Coomassie staining and Western blot analysis with a GFP monoclonal antibody (Clontech). ECL development was carried out as per the manufacturer's instructions (Amersham Pharmacia Biotech). Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence: CEF+hTfR cells were grown on glass coverslips to 50% to 75% confluence. The coverslips were washed and incubated in serum-free Opti-MEM media at 37°C for 1 hour. Then 2 μ g of wild-type GFP (Clontech), HAIYPRH-tagged GFP, THRPPMWSPVWP-tagged GFP, or Texas-Red Tf (Molecular Probes) was

applied to cells in serum-free Opti-MEM media for 1 hour at 4°C or 37°C. Cells were washed with Opti-MEM, then fixed in 3% formaldehyde for 30 minutes at 4°C. Alternatively, the cells were acid-washed with 0.2 M glycine-HCl, Ph 2.2, prior to fixation. A GFP monoclonal antibody (Clontech) was used in conjunction with an Oregon-Green Goat Anti-Mouse (Molecular Probes) to augment GFP fluorescence. All slides were counterstained with DAPI (2(4Amidinophenyl)- 6indole carbamidinedihydrochloride) (Sigma). The microscopic slides were mounted in Prolong™ antifade medium (Molecular Probes). Images were captured on an AX70 microscope with Olympus Camera (Olympus, Melville, NY) and analyzed with ESPRIT software (Life Science Resources, Cambridge, England). Final figures were assembled using Microsoft Power Point (Microsoft Corp., Redmond, WA). For colocalization studies, CEF+hTfR were incubated with 2 µg/ml GFP fusion protein and 2 µg/ml of Texas-Red Tf for 1 hour and processed as described above.

Standard Analysis: Purified proteins (transferrin, wtGFP, GFP-HAIYPRH and GFP-THRPPMWSPVWP) were labelled with ¹²⁵I to a specific activity of 1-2 µCi/µg with CPM/µg determined by a gamma counter and Bradford assay. CEF+hTfR cells were plated in duplicate at a density of 7.5x10⁴ cells/well in 24 well dishes and grown overnight. Cells were washed and incubated in serum-free Opti-MEM for 1 hour at 37°C. Cells were placed on ice with the various amounts of labelled protein in a total of 200 µl of cold 0.1% BSP in phosphate buffered saline (PBS). After 1 hour, the unbound protein was removed and cells were washed 4 times with 0.1%

BSA in PBS. 1 M NaOH was added to lyse the cells for determining the bound fraction. Both unbound and bound fractions were counted in a gamma counter and binding affinities were determined using Scatchard analysis. Studies were repeated 3 times and yielded comparable binding affinities for all proteins tested.

Example 1:

The biopanning procedure with chicken embryo fibroblast cells was performed as described above. The procedure used both negative and positive binding steps to isolate specific peptide sequences that bind the hTfR.

After the cells were incubated in serum-free media Opti-MEM at 37°C for 1 hour to remove Tf found in the serum, ice-cold serum-free Opti-MEM media was applied and the cells were held at 4°C throughout the selected process to prevent internalization of the receptor. The original phage library containing 2×10^{11} phage was applied to CEF cells for 2 hours. Unbound phage were transferred to another well of CEF cells for an additional hour, before transferring the unbound phage to a well of CEF+hTfR cells. After extensive washing, the bound phage were removed with low pH buffer and subsequently neutralized. The eluted phage were titered and amplified in E. coli. After each amplification step multiple plaques were selected for sequencing. The amplified eluted phage were applied to CEF cells to begin the biopanning process again. This cycle was carried out 10 rounds for the 7-mer peptide library and 7 rounds for the 12-mer peptide library to achieve significant enrichment of a single sequence above all others. Sequencing of

individual phage plaques allowed for the monitoring of sequence convergence during multiple rounds of biopanning. Phage titers of total phage eluted were determined and were noticed to increase after each round of biopanning. The most prominent sequence selected from the 7-mer library was HAIYPRH (7-mer) while the 12-mer library converged to the sequence of THRPPMWSPVWP (12-mer). There were no other sequences that arose consistently throughout the biopanning procedure.

Phage that did not bind the CEF cells were applied to CEF+hTfR cells and the bound phage were eluted with low pH buffer. The eluted phage were amplified for additional rounds of biopanning. Between each round, the phage were titered and sequenced to monitor convergence of sequence.

Example 2:

As an initial test to determine whether the isolated phage bound to hTfR, a phage binding study was performed. Homogeneous pools of five different isolates from the 7-mer phage and five isolates from the 12-mer phage were each amplified, purified and verified by DNA sequencing. Individually, 10^9 phage were applied to CEF or CEF+hTfR cells. Phage were bound to CEF or CEF+hTfR cells for 1 hour at 4°C, then washed extensively with Opti-MEM to remove unbound phage. Bound phage were eluted with low pH buffer, neutralized, and titered on a lawn of E. coli. Titering each phage on both cell types was repeated three times, and average titers and standard deviations were determined. Considering the 7-mer sequences, it was found that significantly higher titers were

obtained only with HAIYPRH phage bound to CEF+hTfR cells when compared to other phage tested. In studies relating to the 12-mer sequences, it was found that the THRPPMWSPVWP phage had higher titers on CEF+hTfR cells than the other 12 amino acid phage tested.

5 On CEF cells, which do not express hTfR, all phage tested bound at the same low efficiency. A low level of non-hTfR dependent binding is expected, due to interactions between phage coat proteins and the various proteins on the surface of chicken embryo fibroblast cells. Titering studies demonstrated that phage containing either
10 peptide sequence HAIYPRH or THRPPMWSPVWP bound CEF+hTfR cells more efficiently than any other phage tested and that this higher binding depends on the presence of human transferrin receptor.

Example 3:

Competition studies were conducted to determine whether the
15 two phages bound the same region of the hTfR as serum Tf itself. It was found that the titers of HAIYPRH or THRPPMWSPVWP phage bound to CEF+hTfR cells in the presence of various added peptides or Tf were significant. The HAIYPRH phage was competed away to background levels only by the HAIYPRH peptide and not by the
20 scrambled sequence of IRHPHYA. The 12-mer THRPPMWSPVWP phage was only competed by the THRPPMWSPVWP peptide and not by the scrambled 12-mer sequence PWRPSHPVWMPT. Other peptides tested failed to compete away the phage from binding the cells. Interestingly, the binding of either phage was unaffected by the presence of Tf,
25 suggesting that each phage sequence has a different binding site on the hTfR. Due to a synthesis error, a peptide with the sequence

HAIYPNH was also synthesized. Competition studies were completed with this peptide which disclosed no effect on the HAIYPRH phage binding. The result suggests that the 7-mer phage binding depended on the arginine in the original HAIYPRH.

5 **Example 4:**

To evaluate the sufficiency of capacity of the peptides to mediate uptake of carrier protein, GFP fusion proteins were prepared. Immunofluorescence was used to determine if the GFP-peptides fusion constructs were internalized using the following
10 assay. GFP-peptide constructs were cloned with a C-terminal peptide addition of either HAIYPRH (GFP-HAIYPRH) or THRPPMWSPVWP (GFP-THRPPMWSPVWP). These constructs were expressed and purified to greater than 95% by Coomassie staining. Purified proteins were applied to CEF+hTfR cells at 4°C (which prevents endocytosis) or at
15 37°C. Cells were washed with Opti-MEM, fixed and processed as described under the Materials and Methods section above. Alternatively, the cells were washed with low pH buffer prior to fixation. This acid wash determined whether the protein was endocytosed by removing proteins bound at the cell surface.

20 Immunofluorescence microscopy was used to follow binding and internalization of the wtGFP, GFP fusion proteins and transferrin to CEF+hTfR cells. Wild-type GFP was used as a negative control, while Tf conjugated to the Texas-Red fluorochrome was used as a positive control. The conjugation of Texas-Red to Tf has been
25 shown previously not to diminish interaction with the hTfR. In all studies, cell nuclei were counterstained with DAPI.

Immunofluorescence images of the localization of various proteins applied to CEF+hTfR cells at either 4°C or 37° were studied. At 4°C, endocytosis was blocked so that all proteins remain at the cell surface, and an acid wash removes all cell surface bound proteins. When immunofluorescence of the various proteins was studied on CEF+hTfR cells which had been incubated at 37°C for one hour, localization of GFP-HAIYPRH, GFP-THRPPMWSPVWP or Texas-Red Tf was found on cells that had not been exposed to acid wash. The total fluorescence shown could result from both cell surface and endocytosed proteins. There was minimal binding of wtGFP even without an acid wash.

The cells that had undergone a low pH buffer wash to enable identification of proteins that had been endocytosed were evaluated. While wtGFP was unable to be endocytosed into CEF+hTfR cells, both GFP-HAIYPRH and GFP-THRPPMWSPVWP showed a speckled pattern of fluorescence typical of endocytosed ligands. The Texas-Red Tf was readily endocytosed into the CEF+hTfR cells and produced a spotted pattern similar to that seen with the two GFP fusion proteins.

In separate studies at 4°C or 37°C, CEF cells were used for immunofluorescence binding assays and neither of the GFP fusion proteins or transferrin bound or internalized these cells, as was expected, since these CEF cells lack the hTfR. Immunofluorescent internalization studies were also performed with Hela cells and yielded identical result to CEF+hTfR cells.

Example 5:

The phage titering experiments demonstrated that neither peptide sequence competed with Tf or hTfR binding. Co-localization studies were conducted with both GFP-Peptide and Texas-Red Tf constructs. Cells were incubated at 37°C for 1 hour with Texas-Red Tf and either GFP-HAIYPRH or GFP-THRPPMWSPVWP. Cells were acid washed immediately, fixed and stained with DAPI. Images were captured using the appropriate filter and overlaid with images captured with the DAPI filter. Merging GFP, Texas-Red Tf and DAPI images yielded the co-localization images. The fluorescent patterns of the GFP fusion proteins and Tf were identical after acid wash. This result indicated that the GFP-peptides were internalized and bound in the same intracellular compartment as Tf.

Example 6:

Purified transferrin, wtGFP, GFP-HAIYPRH and GFP-THRPPMWSPVWP were labelled with ^{125}I on tyrosine residues to a specific activity of 1-2 $\mu\text{Ci}/\mu\text{g}$. Serial dilutions of labelled proteins were incubated with CEF+hTfR cells on ice in PBS-0.1%BSA in duplicate wells. After 1 hour, the unbound fraction was removed and cells were washed four times. Cells were removed from the well with 1N NaOH. The unbound and bound fractions were counted in a gamma counter and fmoles of bound and unbound were calculated. Scatchard plots were derived by plotting bound versus bound/free of an average value generated by the duplicate wells. A best of fit line was generated using the Excel program (Microsoft Corp.) and the binding affinities were determined by the slope of the plotted

lines. Repetitive trials produced comparable binding affinities. The affinity of Tf was found to be 2.7×10^{-9} , similar to previous reports. The affinity for wtGFP and GFP-HAIYPRH were determined to be nominal at 2.4×10^{-4} M and 3.6×10^{-4} M, respectively. This low
5 affinity of GFP-HAIYPRH was attributed to the 125 I labelling of the tyrosine residue in the peptide, which could block this peptide's interaction with the hTfR. However, GFP-THRPPMWSPVWP was shown to have 2.3×10^{-8} M affinity for CEF+hTfR cells, indicating that its affinity was only 10-fold lower than the native Tf ligand.

10 Peptides containing the sequences HAIYPRH and THRPPMWSPVWP can be used to target viral vectors, as well as proteins, to the endocytic pathway via the hTfR. Competition studies suggest that transferrin, the 7-mer sequence and the 12-mer sequence all bind
15 unique sites on the hTfR, since they each failed to significantly compete with each other for hTfR binding. This finding suggests further advantages for use of these peptides for transduction of therapeutic ligands, since there is no disruption of transferrin's delivery of iron to cells.

Due to the characteristics and expression pattern of the hTfR,
20 ligands specific for this receptor may be used as targeting agents with antigen as well as diagnostic agents such as imaging agents or radioisotopes. It has been shown that early endosomes are essential for the proper endocytosis, sorting and presentation of antigen by major histocompatibility class II. The targeting of
25 antigens to the hTfR enhances antigen entry into the endocytic pathway and boosts antigen presentation.

It is possible to conjugate the peptides of the invention to liposomes or viral vectors containing active agents such as chemotherapeutics. (See Eavarone, et al, "Targeted Drug Delivery to C6 Glioma by Transferrin-coupled Liposomes", Proceedings of the World Biomaterials Congress 2000, (John Wiley and Sons, Inc.) (2000)). Alternatively, chemotherapeutics may be conjugated directly with the peptides of the invention for targeting agents to transferrin receptor-rich cells. Because the peptides of the invention do not interfere with binding of human transferrin to the hTfR, different agents may be administered wherein one conjugate targets the hTfR uses transferrin as the targeting agent and another conjugate targets the hTfR using a peptide of the invention as a targeting agent.

Example 7:

Transferrin receptor binding peptide sequences to adenovirus proteins in accord with the teachings of U.S. Patent 6,312,699, which is incorporated herein by reference in its entirety. As described in example 2 of US Patent 6,312,699, short peptide ligands such as HAIYPRH and THRPPMWSPVWP are fused onto the carboxyl-terminus of the adenovirus fiber protein. Oligonucleotides encoding these amino acid sequences are designed and synthesized and annealed together for cloning into the unique BamHI restriction endonuclease cleavage site in plasmid pTKgpt-3S (cited in example 2 of US Patent 6,312,699). Examples of such oligonucleotides are:

For HAIYPRH:

Sense: 5' GA TCC CAT GCG ATC TAT CCG CGC CAT TAA 3' (Seq. ID No. 10)

Antisense: 5' G ATC TTA ATG GCG CGG ATA GAT CGC ATG G 3'

(Seq. ID No. 11)

5 For THRPPMWSPVWP:

Sense: 5' GA TCC ACC CAC CGC CCG CCG ATG TGG AGC CCG GTG
TGG CCG TAA 3' (Seq. ID No. 12)

Antisense: 5' G ATC TTA CGG CCA CAC CGG GCT CCA CAT CGG CGG
GCG GTG GGT G 3' (Seq. ID No. 13)

10 These oligonucleotides are designed with BamHI cohesive ends that
can be cloned into the BamHI cleavage site developed in Example 2
of US Patent 6,312,699. The specific amino acid sequence added to
fiber in Example 2 was designed to extend the new transferrin
receptor-binding ligand away from the bulk of the fiber protein,
15 increasing its accessibility to the new receptor molecule. The
fiber protein, modified to include a linker and a ligand, could
still form a trimer.

The non-viral ligands can be attached to the carboxyl
terminus of the fiber protein via a peptide linker by expression of
20 a genetically engineered nucleic acid sequence encoding the fiber
protein, linker, and ligand. Alternatively, one could use PCR
mutagenesis to introduce these two sequences into plasmid
pTKgpt-3S, using synthetic oligonucleotides as in example 4 of the
cited patent.

25 **Example 8:**

The Tf receptor binding peptides can be used to enhance

antigen delivery in antigen-presenting cells. These peptide sequences are applied to increase the potency of vaccines, since antigen-presenting cells often take up the antigens contained in vaccines poorly. To enhance antigen delivery and, therefore, antibody and cytotoxic T cell responses, these peptides are chemically coupled to the antigen of interest or prepared as a recombinant protein that contains these Tf receptor-binding peptides. For preparation of the recombinant antigen containing the Tf receptor binding peptide, coupling is accomplished using standard recombinant DNA techniques as in other examples provided (for example, fusions of HAIYPRH and THRPPMWSPVWP to GFP or adenovirus fiber proteins.) The recombinant proteins can be expressed in any number of protein expression systems including bacterial, baculoviral, and mammalian expression systems.

For chemical conjugation of the Tf receptor binding peptides, the peptides are coupled using chemical crosslinkers such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Pierce Chem. Co., Rockford, IL). Obviously, any chemical crosslinker could be used for this purpose. In our applications, we have coupled 10 mg of antigen to a 5 to 30-fold molar excess of SMCC in 50 mM Hepes buffer (pH 7.4) for 1 h at room temperature.

Example 9:

SMCC-modified antigen is purified by gel filtration to remove the unbound crosslinker. Using this particular crosslinker, peptides are prepared with an amino-terminal linker sequence with a cysteine residue followed by a nonspecific linker sequence

(glycine-proline-glycine) to facilitate the coupling reaction. (The leader sequence can change depending on the nature of the crosslinker.) After the leader sequence, the 7- or 12-residue Tf receptor binding peptide is attached. The peptides are added to SMCC-modified antigens at same molar ratio as is used with the cross-linker. The reactions are incubated overnight at room temperature.

Reaction products are separated by gel filtration and the number of cross-linkers and/or peptides coupled to the antigen is determined by MALDI-TOF mass spectrometry. These Tf receptor binding peptide-modified antigens can then be used as a vaccine using standard vaccination protocols.

The advantage of the peptide-coupled antigens is that substantially less antigen will be required for inducing antibody-based responses. Since a number of peptides can be coupled to each antigen molecule, antigenic responses should be dramatically enhanced.

Example 10:

The peptides of the invention may also be coupled with chemotherapeutic agents. Using 2 equivalents of either peptide HAIYPRH or THRPPMWSPVWP or a combination of the two, to one equivalent of methotrexate the peptides of the invention are coupled to methotrexate using the methods of examples 8 and 9. The resulting product is formulated in buffered saline and administered to the patient in sufficient amount to provide a concentration of .3 to 5 μM in the serum when administered intravenously.

Example 11:

The methotrexate bound to the peptides of the invention is prepared as in example 10. However, the methotrexate bound to the peptides is then formulated in liposomal form for intravenous administration. Liposomal compositions may also be administered by mouth or directly to the affected tissue.

Examples of other antineoplastic agents that might be conjugated to the peptides of the invention, either directly or through conjugation to or incorporation in liposomes containing the sequences of the invention, such liposomes containing antineoplastic agents which may be bound to the peptides of the invention, to target cells rich in human transferrin receptors include (but are not limited to) cisplatin, nitrogen mustards (including chlorambucil), ethylenimines, methylmelamines, nitrosoureas (including carmustin, lomustine, etc.) and doxorubicin. The antineoplastic agents would be administered in accord with the methods usually used for the particular agent and disease. However, because of the selective targetting of the agent by the peptides, lower dosage is required. (The lowering of dosage of the antineoplastic agent can be as much as 80%.) Furthermore, because the over-all dosage of the neoplastic agent can be decreased, the active agent can be administered for a longer period of time and more frequently than when the non-targetting agent is employed.

The compositions with the peptides bound to antigens or antineoplastic agents may be administered directly to the involved

tissues. For example, in cases of malignancy of the respiratory tract, the agents may be administred by inhalation. In treating malignancies of the brain or spinal cord, the agents may be administered intrathecally. For oral administration, the peptide-bound agents may be administred in enteric coated dosage forms to prevent destruction in the stomach.

5

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2209
2210
2211
2212
2213
2214
2215
2216
2217
2218
2219
2220
2221
2222
2223
2224
2225
2226